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DURUM WHEAT CULTIVARS' ANTIOXIDANT AND NITRATE ASSIMILATORY ENZYME RESPONSE TO *BIPOLARIS SOROKINIANA* INFECTION

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ABSTRACT

The biochemical responses of the foliar antioxidant and nitrate assimilatory enzymes to Bipolaris sorokiniana infection were studied in resistant (NIDW295 and PDW314) and susceptible (Bijaga Yellow and A-3-901) durum wheat cultivars. 30 day old durum wheat cultivars were inoculated with spore suspension (10^6 ml^{-1}) of Bipolaris sorokiniana and the uninfected plants served as control. The activities of reactive oxygen species (ROS) scavenging enzymes namely superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) were strongly induced, while those of nitrate reductase and nitrite reductase were suppressed in inoculated leaves (48 hrs post inoculation) compared to healthy ones. The SOD, CAT and GR recorded maximum increase of 344%, 74% and 157% respectively in the resistant cultivars, suggesting their induction for detoxifying ROS synthesized during Bipolaris sorokiniana infection. The relatively lower activities of antioxidant enzymes in the susceptible cultivars make them more prone to oxidative injury than the resistant cultivars. The minimum decline in enzyme activities of nitrite and nitrate reductase in resistant cultivars (29.2% and 25.7% respectively) showed adequate nitrogen regulation, while the susceptible cultivars lacked the ability to maintain the nitrogen metabolism under infection. Thus, our study revealed significant differences in antioxidant enzyme and nitrate assimilatory activities between the Durum wheat cultivars, which may serve as a useful tool in selection of resistant cultivars.

KEYWORDS: Bipolaris Sorokiniana, NIDW295, Bijaga Yellow, Durum Wheat Cultivars, Superoxide Dismutase & Nitrate Reductase

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Original Article

INTRODUCTION

Spot blotch caused by *Bipolaris sorokiniana* (Sacc.) is one of the major foliar diseases that limit the cultivation of wheat in warmer and humid regions of the world (Chowdhury et al. 2013). The pathogen causes deterioration in seed quality, reduces yield, germination, seedling emergence and rooting intensity in the subsequent crops (Joshi, 1986). The grain yield reduction due to spot blotch varies from 15.5 to19.6% in general and 20 to 80% depending upon weather conditions, cultivars and pathogen population (Duveiller and Gilchrist, 1994). Emerging newer strains with increased virulence pose a major threat to wheat production and productivity at the global level (Kumar et al. 2016). Although a number of attempts have been made for efficient management of *B.sorokiniana*, by using disease resistant varieties of wheat, efficient crop rotation techniques and biological controlling agents, spot blotch remains a disease of serious concern under warmer areas (Acharya et al. 2011). Therefore there is a need for understanding the mechanism of resistance and the selection of genotypes with durable levels of resistance to spot

blotch.

The plants defense response to infection by fungi is often manifested as the hypersensitive reaction (HR) characterized by production of reactive oxygen species (ROS), lignin deposition and cellwall crosslinking (Bradley et al. 1992; Brisson, et al. 1994), synthesis of pathogen related proteins (Bowles, 1990) along with induction of rapid and localized death of host cells at the site of infection (Ingram, 1978; Tenhaken et al., 1995). The induction of ROS-scavenging enzymes, such as superoxide dismutase (SOD) involved in the dismutation of the O^{2-} radical to molecular oxygen and hydrogen peroxide, catalase (CAT) and peroxidases (POXs), involved in the removal of H_2O_2, is the most common mechanism for detoxifying ROS synthesized during stress responses (Wojtaszek, 1997; Mittler, 2002). Glutathione reductase (GR) activity maintains an enhanced level of reduced glutathione which is involved in maintaining antioxidant capacity. High antioxidant capacity of plants plays a crucial role in inducing resistance to fungal colonization (Waller et al. 2005).

Nitrate reductase (NR) provides a good approximation of the nitrogen level of plants and is associated with growth and plant yield (Srivastava, 1980). Nitrate assimilation is affected by various environmental factors including the host pathogen interaction. The information about the changes in the nitrate assimilatory system of durum wheat cultivars during *Bipolaris sorokiniana* infection is limited. Therefore, this study aimed to determine alterations in the levels of antioxidant enzymes activities of SOD, CAT and GR and nitrate assimilatory enzymes in four durum wheat cultivars with different levels of resistance to spot blotch.

MATERIALS AND METHODS

Plant Material and Experimental Design

Four cultivars of Durum wheat (*Triticum turgidum* spp.Durum) NIDW295, PDW314 (resistant) and BijagaYellow, A-9-301 (susceptible) were selected to study the biochemical response of antioxidant enzymes and nitrate assimilatory enzymes to *Bipolaris sorokiniana* infection. Two different plots, one for inoculated and another for uninoculated (control) were used for evaluating each cultivar under field conditions and followed a randomized complete block design with three replications **(**Mali et al. 2017) Spore suspension of *B. sorokiniana* (10^6 ml^{-1}), isolated from fungal cultures, was sprayed on to the leaves of durum wheat cultivars (30 days after sowing) until run off. Post inoculation (48 hrs) the activities of SOD; CAT, GR, NR and NiR were assessed in the diseased leaves in comparison with the uninoculated plants (healthy leaves) which served as control.

Extraction of Enzyme and Determination of Protein Content

Fresh leaf tissues after collection were processed immediately for enzyme extraction (0˚C- 4˚C) and assay. In order to measure the enzyme activities of SOD, CAT, GR, NR and NiR, 0.5g of leaf tissue was taken per treatment, and ground into fine powder using liquid nitrogen and extracted in 2.0 mL of ice cold buffer. Extraction buffer for CAT and SOD contained 0.05M sodium phosphate buffer of pH 7.0 and pH 7.8, respectively. Grinding buffer for GR included 0.1M Tris –HCl pH 7.8 and 2mM dithiothreitol (DTT). Nitrate reductase (NR) and Nitrite reductase (NiR) enzyme extracts were prepared in 0.1 M phosphate buffer, pH 7.5, containing 10 mM cysteine. The extraction buffers of all the enzymes contained 1mM ethylenediaminetetraacetic acid (EDTA) and 1.5% w/v insoluble polyvinyl polypyrrolidone was added during extraction. After centrifugation of homogenate at 14,000 rpm for 20 min at 4 ˚C, the supernatant was used immediately as source of enzyme for the assay. An aliquot of supernatant was stored at -20˚C for protein analysis which

was determined by Bradford method using bovine serum albumin as standard (Bradford, 1976).

Antioxidant Enzyme Activities

Superoxide Dismutase

The activity of SOD, (EC 1.15.1.1) was assayed photochemically at 560 nm by the Beauchamp and Fridovich method. (Beauchamp and Fridovich, 1971). 3.0 mL of assay mixture contained 20 µL of enzyme extract, L-methionine (10 mM), p-nitrobluetetrazolium chloride (NBT) (33 µM), EDTA (0.66 µM) and riboflavin (3.3 µM) in a 50mM potassium phosphate buffer, pH 7.8. The assay was initiated by adding riboflavin and took place in a glass tube illuminated by a 15W fluorescent lamp at 25˚C for 20 minutes. The increase in absorbance of the blue formazan produced by NBT photo-reduction was measured at 560 nm.A blank was maintained with all the constituents but in the dark. One unit of SOD is defined as the amount of enzyme required to inhibit 50% of the NBT photo-reduction per minute and specific activity is expressed as IU per mg protein.

Catalase Assay

CAT (EC 1.11.1.6) activity was spectrophotometrically determined by Beers and Sizers method (Beers and Sizer, 1952). The reaction mixture contained 2.98 mL of 16.65 mM hydrogen peroxide in 50mM phosphate buffer, pH 7.0 and 20 µL of enzyme extract was used to initiate the reaction. The decrease in absorbance at 240 nm was measured for 5 minutes using the substrate blank.. One unit of CAT is defined as the one µmole of H_2O_2 decomposed per minute at pH 7.0 at 25˚C and specific activity was expressed as µmole min $^{-1}$ mg^{-1} protein.

Glutathione Reductase Assay

GR (EC 1.8.1.7) activity was determined spectrophotometrically by Mavis and Stellwagen method (Mavis and Stellwagen, 1968). The reaction mixture contained 100 µL of 30 mM oxidized glutathione, 1.5 mL of 100 mM potassium phosphate buffer with 3.4 mM EDTA, pH 7.6, 350 µL of 0.8 mM ß-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) and 950 µL of water. The decrease in absorbance at 340 nm on addition of 100 µL of enzyme to reaction mixture was recorded for 5 minutes.. One GR unit is defined as the amount of enzyme that oxidises 1.0 µmole of NADPH per minute at pH 7.6 at 25°C and specific activity is expressed as µmole min $^{-1}$ mg^{-1} protein.

Nitrate Reductase Assay

NR (EC 1.7.1.1) activity was spectrophotometrically determined by the method of Hageman and Reed (Hageman and Reed, 1980). A known weight (140 mg) of fresh tissue was cut into pieces and suspended in screw cap vials containing 3.5 ml of incubation mixture (20 ml of 0.1M phosphate buffer, 20 ml of 5 per cent propanol and 10 ml of 0.2 per cent KNO3). The vials were sealed and kept in dark condition at 30°C for 2h. Nitrite released into the medium was determined by treating 1 ml aliquot with 1 ml each of 1 % sulphanyl amide and 0.02 % N-1- napthyl ethylene diamine hydrochloride. After 20 min, solution is diluted to 5 ml with water and absorbance is measured at 540 nm. Standard curve is prepared by using reagent grade concentrations of nitrite (KNO2) solution. The nitrate reductase activity is expressed as nmoles of nitrite formed per hour per gram fresh weight.

Nitrite Reductase Assay

NiR (EC 1.7.7.1) activity was determined spectrophotometrically by the method of Wray and Fido (Wray and Fido, 1990). The 0.8 ml of reaction mixture contained 0.2 ml of 0.1M phosphate buffer, 0.1 ml of 5mM sodium nitrite, 0.1

ml of 1.5 mM methyl viologen, 50 µl of enzyme and distilled water. The reaction was started by adding 0.2 ml of the 2.5% sodium dithionite reagent and incubated for 10 minutes. The reaction was stopped by vigorously shaking the mixture until the dithionite was completely oxidized and the dye colour disappeared. For determination of nitrite consumed by enzyme 50 µl aliquot of above mixture was made to 2.0 ml using distilled water and 1.0 ml of 1 % sulphanilamide followed by 1 ml of 0.02 % NNED was added and incubated for 15 minutes. Blank was also processed in the similar way except for 50 µl of enzyme was added after the addition of sulphanilamide and NNED and read at 540 nm. The nitrite consumed by the action of enzyme was estimated from the nitrite standard curve. NiR activity is expressed as µmol of nitrite consumed /min and the specific activity as enzyme activity/mg protein.

RESULTS

Superoxide Dismutase

B.sorokiniana infection showed profound effect on SOD activity. Superoxide dismutase activity in the leaves of all the wheat cultivars differed significantly ($p \leq 0.01$) in inoculated (48 hrs post inoculation) and uninoculated leaves (control) as given in Figure 1. The inoculated leaves of resistant NIDW295 and PDW314 cultivars recorded highest SOD activity (8.63 U/mg proteins and 7.16 U/mg proteins respectively) as compared to control leaves (1.94 U/mg proteins and 2.32 U/mg protein respectively). Inoculated leaves of susceptible A-9-301 and Bijaga Yellow cultivars showed least change in SOD activity (1.72 U/mg proteins and 3.26U/mg protein respectively) as compared to control leaves (1.2 U/mg protein and 2.02 U/mg protein respectively). PDW314 and NIDW295 with 344% and 208% increase in SOD activity indicate strong induction of SOD as defense enzyme against *B.sorokiniana* infection whereas A-9-301 and Bijaga Yellow with 43.3% and 61.3% increase in SOD activity reflect their weak response and hence vulnerability to *B.sorokiniana* infection.

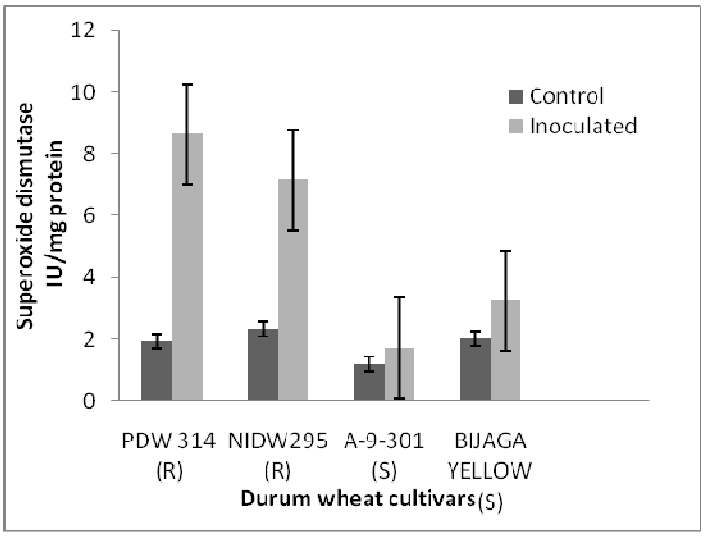

Figure 1: Specific Activity of Superoxide Dismutase in Leaves of Different Durum Wheat Cultivars in Response to Spot Blotch Infection (R)=Resistant Cultivars, (S) = Susceptible Cultivars

Catalase

The catalase activities were significantly ($p \leq 0.01$) greater in the inoculated leaves of all the durum wheat cultivars than those of the control leaves as represented in Figure 2. NIDW295 and PDW314 cultivars recorded significant alteration in catalase activity (703.16 U/mg protein and 623.33 U/mg protein respectively) as compared to control leaves (

404 U/ mg protein and 373 U/mg protein respectively). The response of catalase activity in inoculated leaves of susceptible A-9-301 and Bijaga Yellow cultivars was lower (383.66 U/mg protein and 423.33 U/mg protein respectively) as compared to resistant ones. Amongst all the cultivars the inoculated leaves of NIDW295 showed highest increase in catalase activity (74%) whereas A-9-301 recorded least increase (21.8 %).

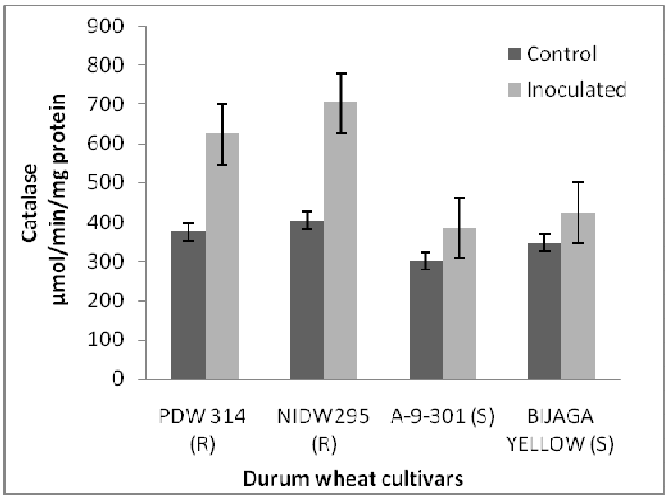

Figure 2: Specific Activity of Catalase in Leaves of Different Durum Wheat Cultivars in Response to Spot Blotch Infection(R)=Resistant Cultivars, (S) = Susceptible Cultivars

Glutathione Reductase

Glutathione reductase activity in the leaves of all the wheat cultivars differed significantly ($p \leq 0.01$) in control and 48 hr after inoculation as given in the Figure 3. *B.sorokiniana* infection elicited variable response in GR activity amongst the cultivars. The inoculated leaves of resistant NIDW295 and PDW314 gave maximum response in glutathione reductase activity (3.423 U/mg protein and 3.233 U/mg protein respectively) as compared to control leaves (1.33U/mg protein and 1.34 U/mg protein respectively) while the Inoculated leaves of susceptible A-9-301 and Bijaga Yellow cultivars showed moderate response (2.06 U/ mg protein and 2.56 U /mg protein respectively) as compared to control leaves (1.37 U/mg protein and 1.03 U/mg protein respectively). NIDW 295 recorded greatest induced activity and increase in Glutathione reductase activity (157.1%) where as Bijaga Yellow showed least increase (86.8 %).

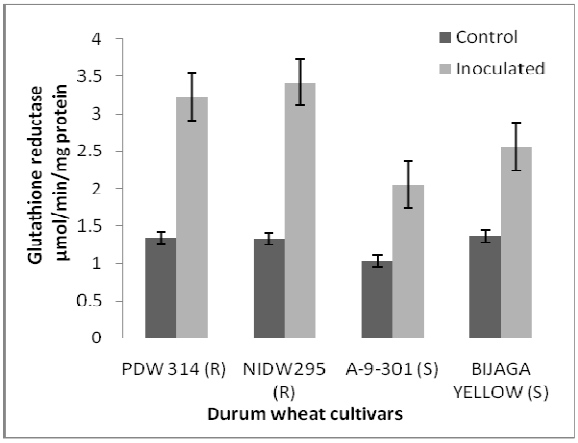

Figure 3: Specific Activity of Glutathione Reductase in Leaves of different Durum Wheat Cultivars in Response to Spot Blotch Infection. (R)=Resistant Cultivars, (S) = Susceptible Cultivars

Nitrate Reductase

Nitrate reductase activity was significantly affected by *B.sorokiniana* infection. Levels of nitrate reductase in all the durum wheat cultivars were significantly lower in inoculated leaves (55.58 nmoles/hr/g) as compared to control (94.47 nmoles/hr/g) as depicted in the Figure 4. Amongst all the cultivars, the nitrate reductase activity in the resistant NIDW295 and PDW314 was significantly higher (100.89 nmoles/hr/g and 87.06 nmoles/hr/g respectively) when compared to susceptible A-9-301 and genotypes Bijaga Yellow (62.28 nmoles /hr/g and 49.84 nmoles/hr/g respectively). Inoculated leaves of susceptible A-9-301 and Bijaga Yellow cultivars showed highest decrease in nitrate reductase activity (39.23 nmoles/g/hr and 28.26 nmoles/g/hr respectively) as compared to control leaves (85.3 nmoles/g/hr and 71.4 nmoles/g/hr respectively). Resistant NIDW295 and PDW314 genotype recorded least decrease in nitrate reductase activity (82.66 nmoes/g/hr and 72.13 nmoles/g/hr respectively) as compared to control leaves (119.13 nmoles/h/hr and 102 nmoles/g/hr respectively). Amongst all genotype inoculated leaves of Bijaga Yellow showed highest decrease in activity (60.4 %) where PDW314 recorded least decrease (29.28 %)

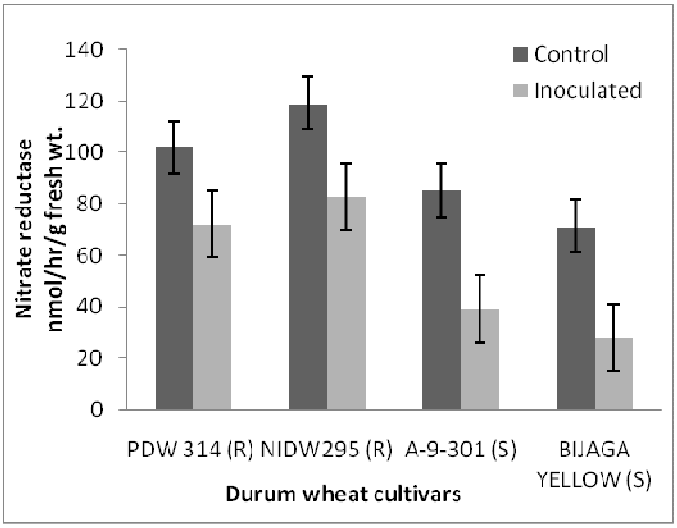

Figure 4: Activity of Nitrate Reductase in Leaves of Different Durum Wheat Cultivars in Response to Spot Blotch Infection (R)=Resistant Cultivars, (S) = Susceptible Cultivars

Nitrite Reductase Activity

In the present study, the NiR activity was decreased significantly ($p \leq 0.01$) in inoculated leaves as compared to control leaves in all the cultivars as represented in the Figure 5. *B.sorokiniana* infection caused higher decrease of NiR activity in susceptible A-9-301 and Bijaga Yellow cultivars than in the resistant NIDW295 and PDW314 ones. Inoculated leaves of susceptible A-9-301 and Bijaga Yellow showed highest decrease in nitrite reductase activity (0.26 U/mg proteins and 0.29 U/mg protein respectively) as compared to control leaves (0.61 U/mg and 0.57 U/mg respectively). Resistant NIDW295 and PDW314 cultivars recorded least decrease in nitrite reductase activity (0.57Umg protein and 0.49 U/mg protein respectively) as compared to control leaves (0.73 U/mg protein and 0.66 U/mg protein respectively). Bijaga Yellow showed highest decrease in NiR activity (61.4%) where as PDW314 recorded least decrease (25.75 %)**.**

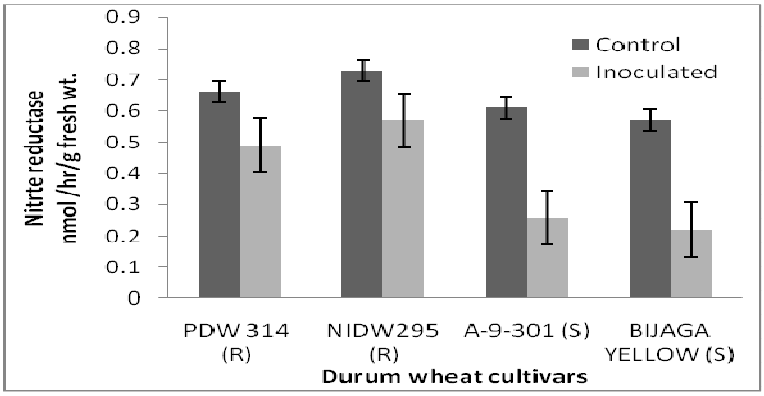

Figure.5: Activity of Nitrite Reductase in Leaves of Different Durum Wheat Cultivars in Response to Spot Blotch Infection (R)=Resistant Cultivars, (S) = Susceptible Cultivars

DISCUSSIONS

Superoxide Dismutase

In the present study, the SOD activities in the inoculated leaves were higher than those of control in all the cultivars, demonstrating SOD as an important protective mechanism against oxidative stress induced by *B.sorokiniana* infection. SOD activity revealed distinct differences in the durum wheat cultivars. The SOD activity increased by 3 - 4.44 folds in the resistant NIDW295 and PDW314 cultivars whereas 1.4-1.6 folds increase was observed in susceptible A-9-301 and Bijaga Yellow cultivars These results are in accordance with the observations of Mahathma et al (2011) and Babitha etal (2002) where higher SOD activity was observed in resistant genotypes compared to susceptible in pearl millet under downy mild dew disease. Within a cell, the superoxide dismutase (SOD) constitutes the first line of defence against ROS (Alscher et al. 2002). Upon confronted by stresses, plants generally produce increased amounts of SODs which act as a part of the innate immunity of plants and is highly correlated with the resistance in plants (Lightfoot, et al. 2016). These results validate that in resistant NIDW295 and PDW314 cultivars SOD optimizes the ROS whereas in susceptible A-9-301 and Bijaga Yellow cultivars, the low SOD activity, was less effective in detoxifying the ROS hence experienced greater damage.

Catalase

Amongst the enzymes involved in the scavenging of the surplus H_2O_2 produced spontaneously or by O^{2-} dismutation via SOD, CAT plays a major role. In the present study, *Bipolaris sorokiniana* infection induced CAT activity in leaves of all the durum wheat cultivars and 1.2-1.6 folds increase was observed in susceptible (A-9-301 and Bijaga Yellow) and resistant (NIDW295 and PDW314) cultivars respectively. Davar et al., (2013) reported a significant 3-fold increase in the CAT activity in resistant sunflower line inoculated with *Sclerotiana sclerotiorum.* The increase in CAT activity may be related to increased oxidative stress tolerance (Yarden et al. 2014). The balance between SOD, CAT and peroxidase activities is important for determining the intracellular level of H_2O_2 and in low concentrations H_2O_2 can act as stress signal. The induced CAT activity in the present study seems to induce tolerance towards *Bipolaris sorokiniana* infection by maintaining H_2O_2 homeostasis.

Glutathione Reductase

In the present study, alterations in the GR activity in leaves of wheat cultivars after *B.sorokiniana* infection was analysed. The GR activity in the leaves of the inoculated cultivars of NIDW295 and PDW314 (resistant) was 2.4 folds greater compared to that of control, confirming their high level of resistance to oxidative stress induced by spot blotch. In contrast, the GR activity only increased slightly in the leaves of susceptible (A-9-301 and Bijaga Yellow) cultivars. GR activity in the resistant plants was increased greatly in the resistant plants of apricot after inoculation with the *Plum pox virus* in comparison with the susceptible (Hernandez et al. 2001). Glutathione reductase recycles glutathione disulfide (GSSG) to GSH and the quick and constant increase in glutathione abundance participates in detoxification of ROS as well as signaling in plant protection against biotic and abiotic stresses (Ghanta and Chattopadhyay, 2015). NIDW295 and PDW314 (resistant) cultivars with higher induced GR activity maintain the balance of the GSH/GSSG cycle and impart the oxidative defence during *B.sorokiniana* infection.

Nitrate Reductase

The primary role of NR in plants is NAD (P) H-dependent reduction of nitrate to nitrite, which is subsequently reduced to ammonium by the nitrite reductase. (Lea, 1993). NR plays an essential role during phytopathogenic interactions by supplying substrates for the synthesis of nitric oxide (NO), a key signal for plant defense responses (Salgado et al. 2013). *B.sorokiniana* infection resulted in reduced nitrate reductase activity in leaves of all durum wheat cultivars. The reduction was more evident in susceptible A-9-301 and Bijaga Yellow cultivars than in resistant NIDW295 and PDW314 cultivars. Similar results were obtained by Sarsenbaev *et al.* in their analysis on consequences of leaf rust infection on the activity of nitrate reductase (NR) of the spring wheat varieties. The maximum decrease in NR catalytic activity may be due to inactivated form of NR or protease degradation of NR triggered by pathogen infection. This decrease would be sufficient to prevent optimal nitrate assimilation in susceptible cultivars and inhibit NO synthesis involved in defense mechanisms.

Nitrite Reductase

Nitrite reductase in plants catalyses the reduction of nitrite to ammonium. The ammonium is then incorporated into amino acids and other nitrogen derived compounds through the glutamine synthetase/glutamine-2- oxoglutarate transaminase system (Lea, 1993). In the present study, nitrite reductase was decreased in *B. sorokiniana* inoculated leaves compared to control leaves and susceptible A-9-301 and Bijaga Yellow cultivars showed maximum decline in NiR activity. Coordination of NR and NiR activities to avoid accumulation of cytotoxic nitrite is very important aspect of regulation of nitrogen metabolism (Gupta and Beevers, 1983). Lower NiR activities in susceptible cultivars may lead to accumulation of nitrite which can be detrimental to the plant.

CONCLUSIONS

The present study revealed higher activities of antioxidant enzymes SOD, CAT and GR in the defence response of resistant NIDW295 and PDW314 cultivars against *Bipolaris sorokiniana* infection compared to susceptible A-9-301 and Bijaga Yellow cultivars. Slight decrease in activities of NR and NiR enzymes observed in resistant cultivars indicate the ability of cultivars to efficiently regulate nitrogen metabolism under *Bipolaris sorokiniana* induced stress. Thus significant differences in antioxidant enzyme and nitrate assimilatory activities were observed between the resistant and susceptible cultivars which may serve as an useful tool in selection of resistant cultivars. However, further profiling of isozymes of the antioxidant enzymes are needed to confirm the genes unique to resistant durum wheat cultivars and thus may provide

useful markers for spot blotch resistance.

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